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Phylis M. Rhinehart, US Army Medical Research and Materiel Command, MCMR-RMI-S [70-1Y] DCS Info. Mgmt. Ft. Detrick, MD

AD_____

CONTRACT NO: DAMD17-91-C-1107

TITLE: PROLONGED PRESERVATION OF HUMAN PLATELETS FOR COMBAT CASUALTY CARE

PRINCIPAL INVESTIGATOR: Stephen A. Livesey, M.D., Ph.D.

CONTRACTING ORGANIZATION: LifeCell Corporation
3606 Research Forest Drive
The Woodlands, Texas 77381

REPORT DATE: April 15, 1994

TYPE OF REPORT: Midterm Report (Phase II)

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	4/15/94	Midterm report 04/93 - 04/94

4. TITLE AND SUBTITLE
Prolonged Preservation of Human Platelets for Combat Casualty Care

5. FUNDING NUMBERS
Contract No:
DAMD17-91-C-1107

6. AUTHOR(S)
Jerome Connor, Ph.D., Project Manager and
Stephen Livesey, M.D., Ph.D., Chief Scientific Officer

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
LifeCell Corporation
3606 Research Forest Drive
The Woodlands, Texas 77381

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Midterm Report for Phase II Research

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Specific Authority
Distribution authorized to DOD Components only, 4/15/94.
Other requests for this document shall be referred to: The above address

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This report describes a method for extended storage of human platelets. The platelet storage is performed at 4°C or with cryopreservation to eliminate the bacterial contamination problems associated with conventional storage. To abrogate platelet storage lesions which occur during the 4°C storage, the cells are treated with second messenger effectors which stabilize the platelets by inhibiting the biochemical events which lead to storage lesion thus, the platelets remain active. Platelets treated with this stabilization mixture display activity profiles, following 6 days of storage at 4°C, equivalent to fresh platelets (i.e. 100% recovery of activity). In contrast, platelets stored at 4°C in the absence of the stabilization mixture yield <60% recovery of cell number, ~10% response to agonist stimulation and ~10% response to hypotonic stress. Moreover, platelets stored by conventional methods for 5 days, the maximal allowable, display only between 50%-85% agonist response and ~60% hypotonic response. Platelets, treated with the stabilization mixture and cryopreserved via the conventional DMSO method, yield >95% recovery of cell number, ~70% response to collagen stimulation and ~75% response to hypotonic stress, as compared to fresh platelets. The results of these experiments demonstrate that the implementation of this method allows platelets to be stored at 4°C or via cryopreservation for extended periods, without loss of activity.

14. SUBJECT TERMS
DTIC QUALITY INSPECTED 8
Platelet Preservation; Cryopreservation

15. NUMBER OF PAGES
42

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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Prolonged Preservation of Platelets for Combat Casualty Care

LifeCell Corporation
3606 Research Drive
The Woodlands, TX 77381

Principal Investigator: Stephen A. Livesey, M.D., Ph.D.
Exectutive Vice President and
Chief Scientific Officer

Project Manager: Jerome Connor, Ph.D.
Project Manager
Cell Projects

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Background

During the first six months of this project we generated in vitro data on the application of specific second messenger effectors to extending the storage of platelets at 4°C. These modifiers affect specific second messenger pathways which in turn render the platelets less susceptible to storage lesions during storage at 4°C. The second messenger systems and the specific modifiers which effect these systems are as follows:

1) Amiloride: Amiloride is an inhibitor of the Na^+-H^+ exchanger.

2) Adenosine: Adenosine stimulates the production of cyclic AMP.

3) Sodium Nitroprusside (NP): NP stimulates the production of cyclic GMP.

4) Amantadine: Amantadine is a phospholipid intercalating molecule which affects membrane fluidity.

5) Flurbiprofen: Flurbiprofen is an inhibitor of the cyclooxygenase enzyme and thus blocks this arm of the arachidonic acid cascade.

6) Quinacrine: Quinacrine serves as a phospholipase A_2 inhibitor, blocking the breakdown of the lipids which provide the source of the arachidonic acid cascade.

7) Dipyridamole: Dipyridamole inhibits both the cyclooxygenase and the lipoxygenase enzymes of the arachidonic acid cascade.

All of these platelet effectors have been demonstrated to inhibit agonist induced aggregation. More importantly, the inhibition provided by each of these inhibitors is reversible following removal of the effector by washing the platelets. Employing this inhibitor system, we demonstrated that upon addition of these modifiers, both independently and in combination, platelets were less susceptible to storage lesions during storage at 4°C and that upon removal of the effectors the

platelets displayed normal aggregation physiology.

A second experimental approach evaluated during the initial six month period was the use of a defined storage buffer to replace the plasma traditionally used as a storage media for platelets. We developed a buffer formulation in which platelets, treated with the above discussed modifiers, could be successfully stored at 4°C. A significant component of this storage buffer was the inclusion of heparin and apyrase. Apyrase is an ATPase/ADPase which contributes to the inhibition of ADP-induced activation of platelets during storage. An important feature of the storage buffer is the ability to demonstrate efficacy of the storage system. Since the platelets are stored at a concentrated cell number in the storage buffer system, the cells can be diluted into fresh plasma following the storage period at 4°C, thereby reversing the inhibitors' effective concentrations. The platelets can then be evaluated for activity as a measure of the ability to resist storage lesions. Employing this wash buffer in combination with second messenger effectors, we demonstrated that platelets stored at 4°C for greater than ten days retained the ability to aggregate upon stimulation with the agonists ADP and collagen.

Preliminary studies were undertaken during the initial stage of this research program to determine the effectiveness of employing the second messenger effector system, developed for 4°C storage to the extended storage of platelets by cryopreservation or freeze-drying. Initial experiments using second messenger modifiers in combination with 6% DMSO demonstrated that platelets which had undergone a freeze-thaw cycle retained the ability to aggregate in response to agonist stimulation. In addition, platelets subjected to conventional freeze-drying techniques showed some activation response following rehydration.

The data accumulated during the initial six months of this project clearly indicated the success of employing the second messenger effectors as a means to stabilize the platelets during storage both at 4°C and by freezing. During this second six month period we will demonstrate the further development of using second messenger modifiers to stabilize the platelets during storage, such that the stored platelets display activity levels similar to fresh platelets. This will be achieved by the application of additional second messenger agents and the further evaluation of effector combinations which best stabilize the platelets. Furthermore, additional in vitro evaluation criteria will be used to better demonstrate the platelets' activity profile following storage.

4°C Storage of Platelets

Platelet Processing

During the previous report, we presented the development of a wash buffer storage system which removed the plasma from the platelet preparation and replaced it with a defined storage buffer. Included in this buffer was the ADPase enzyme, apyrase, which contributed to inhibiting storage lesions during 4°C storage. While this buffer was as effective as the autologous plasma, the inclusion of an agent (apyrase) not approved for in vivo use presented a problem in the ensuing transfusion step necessary for use of the final platelet product. Moreover, the inclusion of apyrase in the storage buffer was demonstrated to be essential, removal of this enzyme yielded a preparation which was susceptible to storage lesions during 4°C storage (data not shown). Therefore, while important data concerning the application of second messenger modifiers was develop with this storage buffer, it was decided that further development of extended storage of platelets using this buffer system was inappropriate. Thus, the continued evaluation of using second messenger effectors to control platelet storage lesions during extended storage at 4°C was performed using autologous plasma. This rendered the platelets available for direct transfusion following storage.

A second point of consideration, which concerns the processing of platelets with regards to the development of an applicable end product, arose from our continued discussion with the Gulf Coast Regional Blood Bank and the Red Cross. Our current method of acquiring platelets, both for small research quantities and unit preparations, involves adding amiloride to the whole blood followed by generation of PRP. At this point, the additional second messenger effectors (adenosine and NP) are added to the PRP and the platelets are pelleted by centrifugation. The majority of the plasma is

expressed and the platelet pellet is resuspended at a 1/10 volume. This protocol presents a problem for the Blood Bank and other blood procurement agencies, which follow strict procedures for the aquisition and processing of whole blood and blood components. The concern is the effect of 1) amiloride on the erythrocytes, since it is added to whole blood, and 2) adenosine and NP on the plasma fraction, since they are added to the PRP. In other words, if our current protocol for the processing of platelets for 4°C storage is initiated, the other blood components (erythrocytes, plasma, plasma factors) would be compromised, and thus unavailable for ancillary transfusion.

Based on this new information, an alternative protocol for the acquisition and processing of platelets has been developed and tested in order to overcome the aforementioned problems. By eliminating the need to add the second messenger effectors to the whole blood or PRP, the other important blood components are available for transfusion. The new method for acquisition of a platelet preparation for storage at 4°C is as follows:

1) Whole blood is collected into ACD and PRP is generated by centrifugation following conventional procedures.

2) PRP is centrifuged and the plasma is expressed such that the platelet pellet retains 1/10 volume of plasma.

3) The second messenger agent mixture (approximately 1/100 volume of the platelet volume) is added to the platelets with gentle mixing.

4) The stabilized platelet/effector mixture is stored at 4°C without agitation.

To demonstrate the effectiveness of this protocol, a comparison of the original method and the alternative procedure was performed and is shown in Table 1 and Table 2. In this experiment the second messenger effector mixture of amiloride, adenosine and NP [triple complex (TC)] was employed, based on the effectiveness of this solutions in earlier experiments. In all of the following

experiments the effector mixture consisting of amiloride, adenosine and NP is termed the triple complex or TC. The concentration of these effectors is based on extensive experimentation that is detailed in the "Prolonged Preservation of Human Platelets for Combat Casualty Care" six month report submitted on October 15, 1993. Unless otherwise stated, the conceneration of the TC reagents is amiloride - 1 mM, adenosine - 0.1 mM, and NP - 25 uM.

Table 1. Effects of the Point of Addition of Second Messenger Agents.

	% of PRP at t=0[5]											
Conditions	ADP Aggregation time (days)				Collagen Aggregation time (days)				HSR time (days)			
	0	2	6	10	0	2	6	10	0	2	6	10
Control	75	50*	18*	6*	94	71*	41*	23*	80	36	0	0
TC[1,2]	50	88	56	0	94	100	71	0	100	31	0	0
TC[3]	50	88	50	13	88	100	71	29	100	52	7	0
TC[4]	56	69	63	25	94	100	59	35	78	53	12	0

[1]TC is the Triple Complex consisting of 1mM amiloride, 0.1mM adenosine and 25uM NP.
[2]Amiloride added to the whole blood; adenosine and NP added to the PRP.
[3]Amiloride, adenosine, and NP added to the PRP.
[4]Amiloride, adenosine, and NP added to the platelet pellet.
[5]All results are normalized to fresh processed platelets at t = 0 rather than parallel processed controls which are shown as the first line in each Table.
*Spontaneous activation

In order to better evaluate platelet function and the effects of 4°C storage on platelet recovery, a new assay has been incorporated into our experimental methods. In addition to the agonist-induced aggregation, the platelets were examined for the ability to recover from hypotonic stress. This assay, termed hypotonic stress response (HSR), is a photometric measurement of the platelets' ability to overcome the addition of a hypotonic solution. This activity reflects cell function (i.e. a functional membrane water pump) and is indicative of platelet recovery following

storage. HSR has been demonstrated to be an important indicator of the platelets' ability to survive in circulation following transfusion. Therefore, HSR represents a crucial parameter for evaluating the platelet biochemistry following extended storage at 4°C.

Table 2. Effects of the Point of Addition of Second Messenger Agents.

	% of PRP at t=0											
Conditions	ADP Aggregation time (days)				Collagen Aggregation time (days)				HSR time (days)			
	0	3	7	10	0	3	7	10	0	3	7	10
Control	100	23*	0	0	94	47*	0	0	94	18	8	0
TC[1#]	59	82	35	12	100	94	53	18	87	36	14	1
TC[2]	65	82	41	18	100	100	71	24	82	71	12	3

[#]TC is the Triple Complex consisting of 1mM amiloride, 0.1mM adenosine and 25uM NP.
[1]Amiloride, adenosine, and NP added to the PRP.
[2]Amiloride, adenosine, and NP added to the platelet pellet.
*Spontaneous activation

The results of these experiments demonstrate the following:

1) The addition of the second messenger effectors to the platelet pellet was as effective as an addition to the whole blood or to the PRP.

2) Control platelets stored at 4°C displayed storage lesions (i.e spontaneous aggregation) and loss of activity at very early time points (1-2 days).

3) Platelets stored at 4°C with the TC solution showed a loss of the ability to aggregate in response to agonist stimulation beginning at six days.

4) HSR for platelets stored with the TC mixture declined at an earlier time than aggregation (< 6 days)

5) Platelet cell number for control platelets was less than 60% by day two, while the TC treated platelets showed no loss of platelet cell number, even at 10 days (data not shown).

Effects of the Triple Complex Mixture with Additional Inhibitors

Previous experiments demonstrated the utility of using the basic second messenger effector mixture consisting of amiloride, adenosine and NP, termed triple complex (TC). The results of these studies showed that the TC solution completely abrogates the agonist-induced aggregation of platelets and this effect is readily reversible following removal of the effectors. More importantly, the TC mixture enables platelets to be stored at 4°C for extended periods of time without the loss of cell number. The platelet population, though, did not display an activity level on the same order as fresh platelets at these longer storage periods but instead, displayed decreasing activity with time. These results demonstrate the concept that stabilization of the second messenger systems renders the platelets capable of surviving 4°C storage with the potential to respond to agonist stimulation, although the effect was lost over extended time periods. Thus, in order to further control the second messenger biochemistry of platelets, additional platelet biochemistry effectors were examined for the potential to reversibly inhibit platelet activation, in an effort to enhance the effects of the TC mixture.

The additional second messenger agents to be evaluated in conjunction with the TC solution were the arachidonic acid cascade inhibitors, which were previously demonstrated individually to effectively block agonist stimulated activation. These agents are quinacrine, flurbiprofen and dipyridamole. These experiments were performed initially by adding amiloride to the whole blood followed by generation of PRP. The platelets are then pelleted and resuspended in the storage buffer with the indicated modifier combination as described in the previous report. The platelets are analyzed for agonist-induced aggregation in the presence of the effectors, following a wash step at 0 hours or after storage for 24 hours at 4°C. The cells are stored at 1/10 volume and diluted up to the original volume at the indicated time point, which effectively reverses the inhibitors' effect. Table 3

shows the ability of these new second messenger agents in combination with the TC mixture to reversibly inhibit platelets.

Table 3. Effects of Arachidonic Acid Metabolism Effectors.

Conditions[1]	% of PRP at t=0					
	ADP Aggregation time (days)			Collagen Aggregation time (days)		
	0	0[2]	1	0	0[2]	1
Quinacrine 0	0	25	13	0	67	28
5nM	0	25	38	0	67	56
50nM	0	25	37	0	44	61
.5M	0	6	25	0	38	28
Flurbiprofen 0	0	6	0	0	63	13
10nM	0	6	0	0	56	19
.1M	0	19	0	0	69	31
1M	0	0	0	0	44	19
10M	0	0	0	0	63	13
Dipyridamole 0	0	22	16	0	78	61
10M	0	11	17	0	67	33
.1mM	0	17	33	0	22	44
1mM	0	22	22	0	28	33

[1]All samples contain amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[2]Samples at t = 0 following a wash step.

The results from these experiments demonstrate the following:

1) Quinacrine and dipyridamole both effectively and reversibly inhibited platelet activation.

2) Quinacrine and dipyridamole both enhanced the TC stabilization of platelets during 4°C storage.

3) Flurbiprofen in combination with TC was not reversible in controlling platelet activation and thus was ineffective as an additional component of the storage solution.

The conclusion of this experimental series is that the inclusion of quinacrine and dipyridamole with the TC mixture represents a viable method to enhance the stabilization of platelets during 4°C storage. This supports the hypothesis that by controlling the various pathways of activation by manipulation of the second messenger cascades, the storage lesions which occur at 4°C can be stabilized, thus allowing the platelets to be stored at this temperature.

In order to evaluate additional compounds which may affect platelet activation, the following compounds were examined individually in PRP for the ability to reversibly inhibit agonist-induced aggregation and the results are outlined in Table 4.

1) Nifedipine: Nifedipine is a calcium channel blocker. Blocking the uptake of calcium into platelets should block the agonist-induced activation of these cells.

2) Droperidol: Droperidol is a an anesthetic with anti-thrombotic effects.

3) Verapamil: Verapamil is a calcium channel blocker.

4) Sulfinpyrazone: Sulfinpyrazone is an in vivo anti-thrombotic agent which blocks platelet activation.

5) Ketorolac: Ketorolac is a non-steroidal aspirin substitute which effects prostaglandin biochemistry.

6) Ticlopidine: Ticlopidine is an in vivo platelet aggregation inhibitor.

Table 4. Effects of Additional Second Messenger Agents.

Conditions	% of PRP at t=0	
	ADP Aggregation	Collagen Aggregation
Nifedipine		
.1mM	100	94
1mM	100	82
Droperidol		
1mM	100	94
Verapamil		
1M	100	94
.01mM	93	94
Sulfinpyrazone		
1mM	100	100
10mM	100	100
20mM	100	100
Ketorolac		
1mM	88	100
10mM	47	68
10mM - wash[1]	94	100
Ticlopidine		
1mM	100	100
2.5mM	52	100
3mM	41	50
5mM	0	25
5mM - wash[1]	94	100

[1]Platelet aggregation following a wash step to remove the inhibitors.

Table 4 demonstrates that the calcium channel blockers were ineffective in inhibiting agonist-induced

aggregation. In contrast, both Ketorolac (Ktc) and Ticlopidine (Tcp) displayed the ability to inhibit platelet activation and this effect was readily reversible following washing. Thus, these two new second messenger agents can be tested for the ability to augment the stabilization of platelet biochemistry, in conjunction with the TC mixture, in formulating a storage condition.

Evaluation of the Five Inhibitor Complex Mixture (FC)

The data presented above employing the TC mixture to stabilize the platelets during storage at 4°C indicated that while this method was effective in controlling storage lesions, the protective effect of these second messenger modifiers began to decline over time, thus limiting the length of 4°C storage. In an effort to overcome this time dependent decline in platelet activity, additional second messengers effectors, which target different biochemical systems, in platelets were examined for the ability to extend the storage life of platelets stored at 4°C and increase the recovery of these platelets. Table 5 demonstrates the platelet activity of platelets stored at 4°C with the TC solution and the enzyme inhibitors quinacrine (quin) and dipyridamole (dpm). Both quinacrine and dipyridamole were shown previously to reversibly inhibit agonist-induced aggregation in conjunction with the TC mixture. In these experiments, both enzyme inhibitors were evaluated for the ability to block storage lesions during the 4°C storage of platelets and to increase the amount of recoverable activity as compared to the TC solution alone.

Table 5. Effects of Arachidonic Acid Metabolic Effectors in Conjunction with the TC Mixture

Conditions	% of PRP at t=0								
	ADP Aggregation time (days)			Collagen Aggregation time (days)			HSR time (days)		
	0	1	3	0	1	3	0	1	3
Control	82	12	0	100	12	0	74	8	0
TC[1]	59	82	35	100	88	76	92	61	7
TC w/dpm	59	53	41	100	94	82	55	79	15
TC w/quin	76	88	47	100	94	94	100	82	31
FC[2]	65	88	65	94	88	94	79	78	38

[1]TC is the Triple Complex consisting of 1mM amiloride, 0.1mM adenosine and 25uM NP.
[2]FC is the Five Agent Complex consisting of the TC (concentration as described above) with dpm (20uM) and quin (0.1uM).

The results of these experiments yield the following conclusions:

1) Individually, quinacrine and dipyridamole, added to the TC mixuture, improved the post-storage activity of platelets as compared to the TC mixture.

2) In combination, quinacrine and dipyridamole, increased the post-storage activity profile more than these agents added alone.

3) The combination of the TC solution with quinacrine and dipyridamole represents the best combination tested thus far for stabilizing platelets during 4°C storage.

This new second messenger effector mixture which consists of the TC with the addition of two new modifiers, quinacrine and dipyridamole, yields a five agent complex (FC) mixture. Table 4 demonstrates the effective inhibition of agonist-induced aggregation by a new series of platelets reactive agents. The results of those experiments showed that both ketorolac and ticlopidine represent viable platelet modifiers to analyze in the current storage mixture. Therefore, ketorolac was added to the FC mixture and analyzed for the ability to enhance the stabilization effect demonstrated by the FC mixture. Table 6 displays the results of the experiment which compared 1) the TC mixture,

2) the FC mixture and 3) the FC mixture with ketorolac.

Table 6. Effects of Ketorolac Addition to the TC and FC Mixtures

	% of PRP at t=0								
Conditions	ADP Aggregation time (days)			Collagen Aggregation time (days)			HSR time (days)		
	0	1	3	0	1	3	0	1	3
Control	94	71	18	100	94	53	81	32	16
TC[1]	41	53	35	94	100	88	80	50	8
TC w/dpm	24	31	29	88	82	65	58	45	25
TC w/quin	24	52	35	94	94	71	70	32	32
FC[2]	35	46	24	94	94	71	53	43	22
FC w/ktc[3]	35	47	24	71	59	29	68	23	10

[1]TC is the Triple Complex consisting of 1mM amiloride, 0.1mM adenosine and 25uM NP.
[2]FC is the Five Agent Complex consisting of the TC (concentrations as described above) with dpm (20uM) and quin (0.1uM).
[3]The Five Agent Complex (concentrations as described above) with ktc (10mM).

The results from Table 6 show that the addition of ketorolac to the FC mixture was detrimental to the post storage activity. This may be due to a irreversible effect of ketorolac during the storage interval or possibly due to an adverse interaction with one or more of the other second messenger agents. Regardless, the addition of ketorolac to the FC does not represent a viable approach to further increasing the activity of platelets stored at 4°C.

An additional second messenger effector, which showed the ability to reversibly inhibit agonist-induced platelet aggregation, was ticlopidine (Table 4). Ticlopidine was added to the TC mixture and used in the storage buffer for the platelets at 4°C. Table 7 shows the results of the activity parameters of the platelets kept at 4°C in this storage buffer.

Table 7. Effects of the Addition of Ticlopidine.

Conditions	% of PRP at t=0								
	ADP Aggregation time (days)			Collagen Aggregation time (days)			HSR time (days)		
	0	1	5	0	1	5			5
Control	100			100					5
TC[1]	20	40	27	88	100	63			47
TC w/tcp[2]	27	27	13	88	100	81			79

[1]TC is the Triple Complex consisting of 1mM amiloride, 0.1mM adenosine and 25uM NP.
[2]TC (concentrations as described above) with tcp (1.5mM).

The results of these experiments indicated that the addition of ticlopidine to the TC solution enhances the ability of the platelets to resist storage lesions during the 4°C incubation period. The inclusion of ticlopidine increased the recovery of both the collagen induced aggregation and more importantly increased the hypotonic stress response two fold over the TC mixture alone. This data demonstrates that increased stabilization of platelets at 4°C can be achieved by affecting additional second messenger pathways of the platelets.

Based on the observations that ticlopidine can enhance the post storage activity profile of platelets, this agent was next examined in conjunction with the FC mixture, which was previously shown to be more effective than the TC mixture. In addition, earlier experiments employing the wash buffer method demonstrated that the inclusion of heparin in the storage solution improved the recovery of the platelets following 4°C storage, thus heparin was also tested in the FC mixture for its ability to block storage lesions and increase the platelet recovery. In these experiments ticlopidine and heparin were added, alone or together, to the FC mixture and analyzed for the effects on 4°C storage of the platelets. The results of these experiments are shown in Table 8.

Table 8. Effects of the Addition of Heparin and Ticlopidine to the FC Mixture

Conditions	% of PRP at t=0								
	ADP Aggregation time (days)			Collagen Aggregation time (days)			HSR time (days)		
	0	1	3	0	1	3	0	1	3
Control	50	56	31	94	94	71	86	100	62
FC[1]	13	13	13	88	82	71	74	89	81
FC w/tcp[2]	13	13	13	82	76	88	81	100	100
FC w/Hep[3]	38	44	38	100	71	71	92	100	26
FC w/tcp/Hep	25	44	25	82	82	71	85	92	93

[1]FC is the Five Agent Complex consisting of the TC (concentrations previously described) with dpm (20uM) and quin (0.1uM).
[2]Five Agent Complex (concentrations as described above) with tcp (1.5mM)
[3]Five Agent Complex (concentrations as described above) with Hep [0.13mg/ml (20u/ml)]

The experimental results of this analysis are as follows:

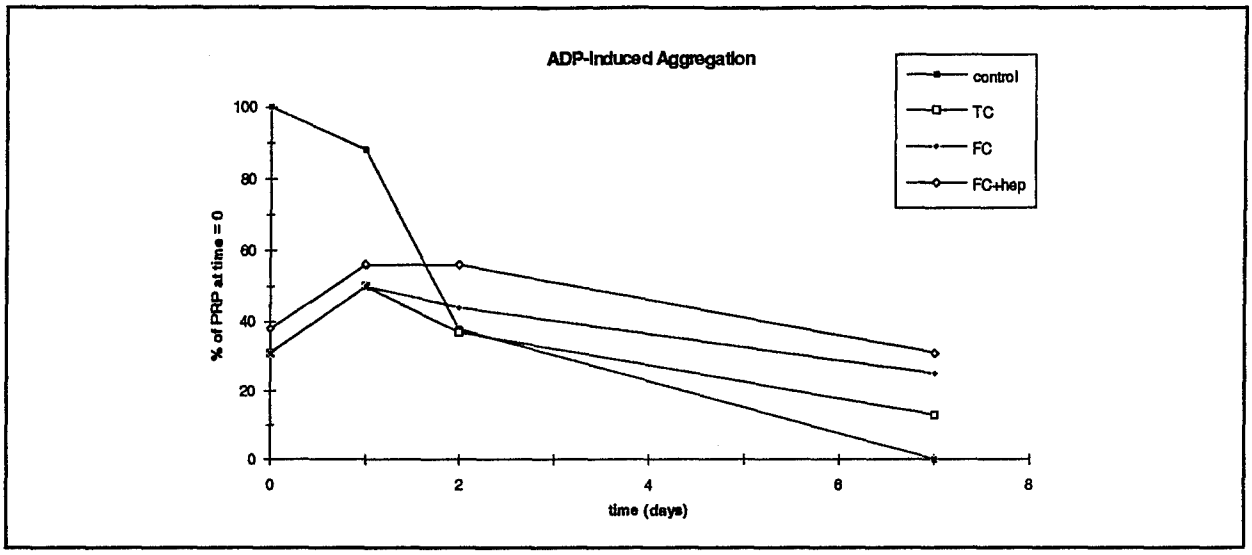
1) The addition of ticlopidine to the FC mixture enhanced the post storage activity profile.

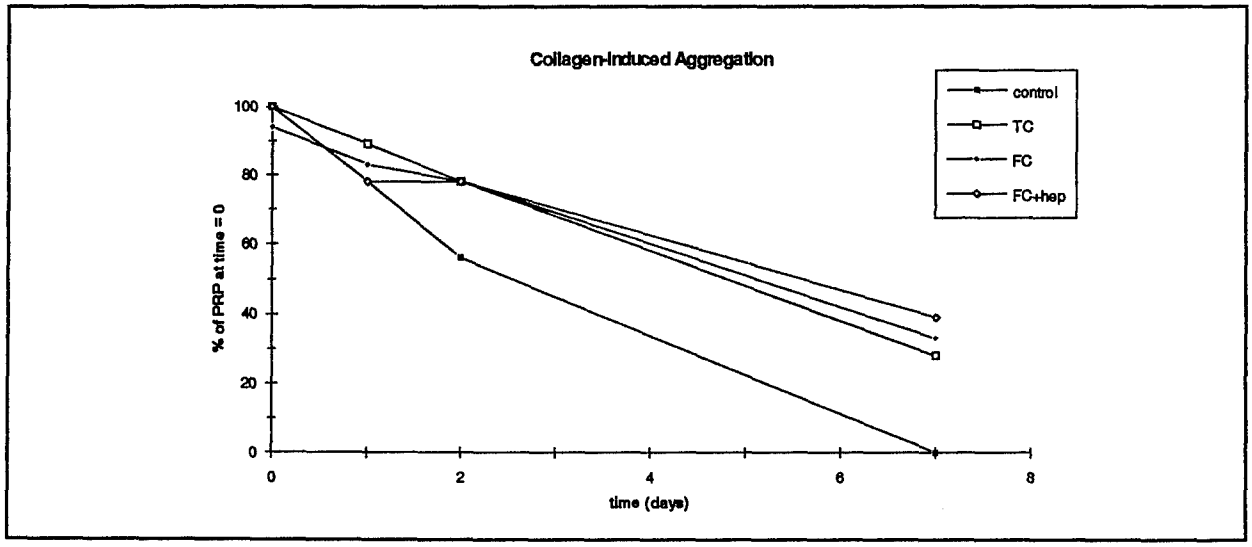
2) The addition of heparin also increased the activity parameters of the platelets stored at 4°C.

3) The combination of ticlopidine and heparin enhanced the FC mixture in stabilizing platelets against storage lesions.

These results demonstrate that control of the second messenger systems of platelets enable these cells to be stored at 4°C without spontaneous activation, without storage lesions and without any loss of cell number. At the time intervals analyzed (0-3 days) the platelets' activity profiles, following storage, were similar to those of fresh platelets immediately following acquisition. In order to develop an applicable 4°C storage system, though, the use of a second messenger stabilizing solution needs to be evaluated at longer time intervals to demonstrate that 4°C stabilization of the platelets can be maintained over longer times.

Figure 1. Effects of the TC and the FC Mixtures on Extended Storage of Platelets.





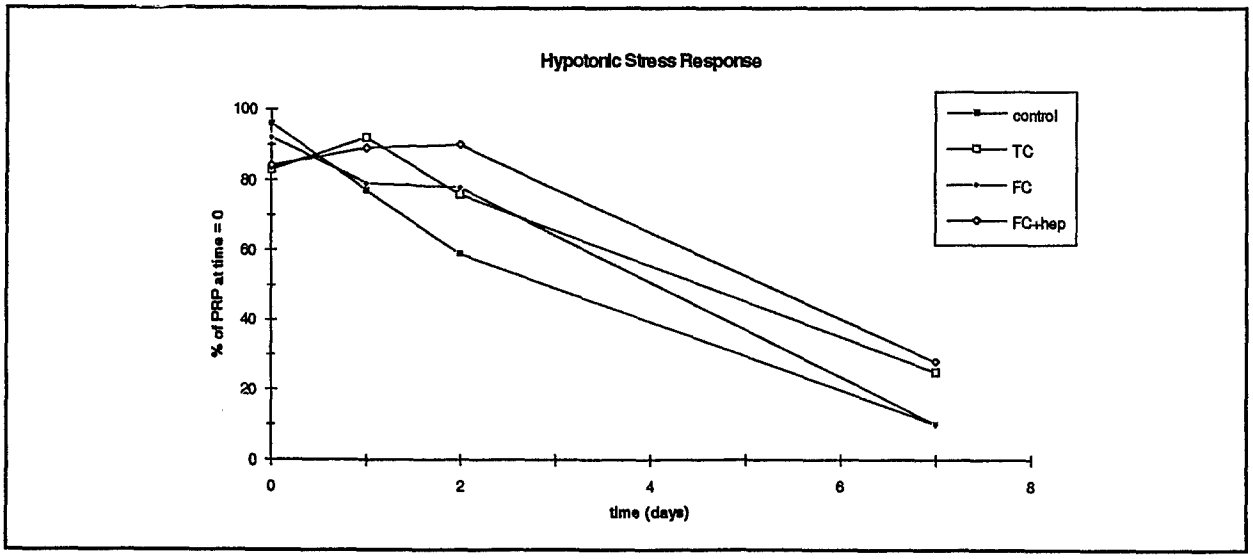
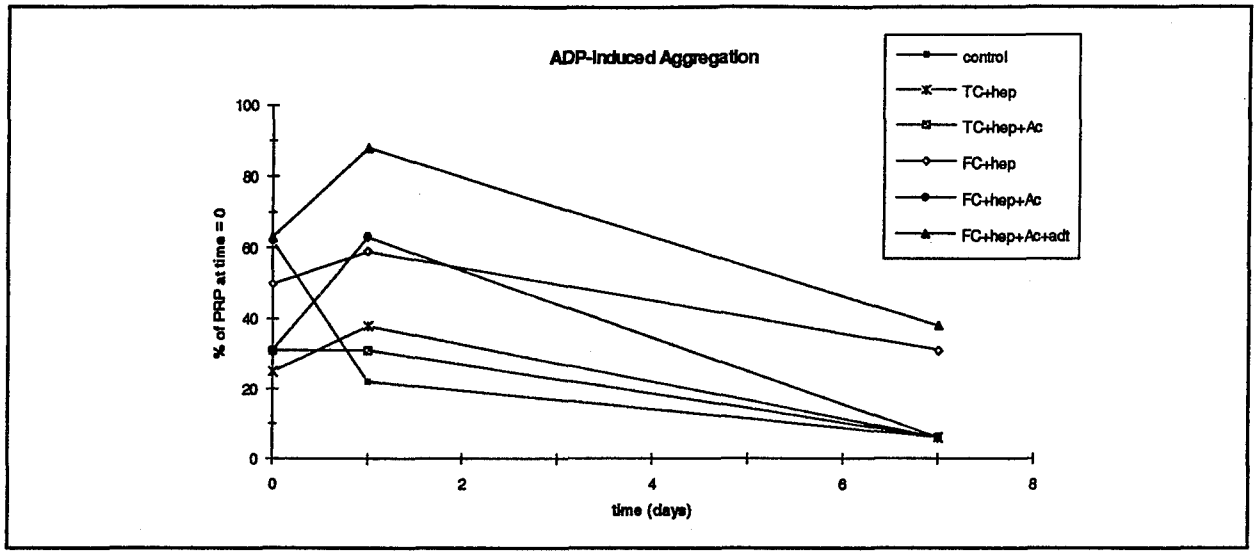


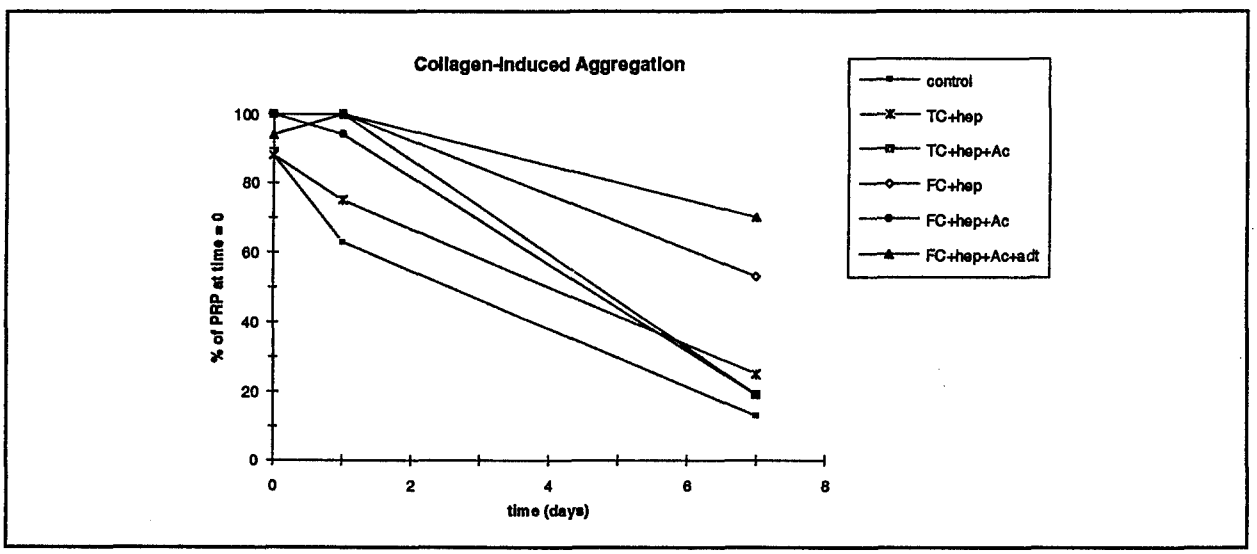
Figure 1 demonstrates the analysis of the TC mixture, the FC mixture and the FC mixture with heparin at extended time intervals (concentrations as described for Table 8).

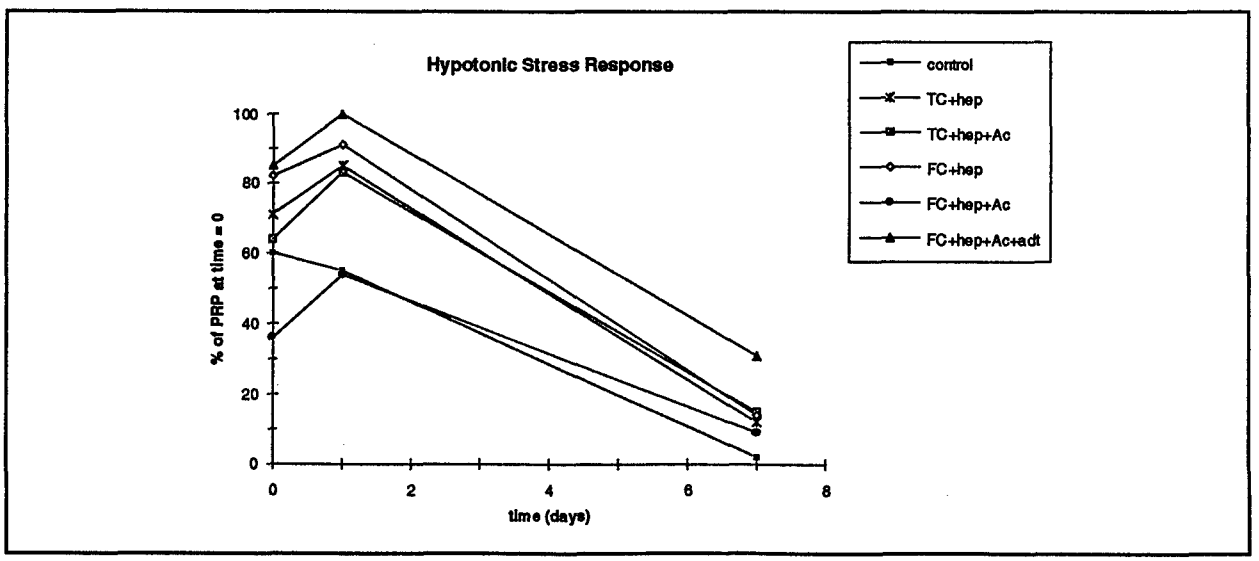
As shown in the earlier results, the addition of second messenger effectors stabilized the platelets, thereby decreasing storage lesions and increasing the platelets activity parameters following 4°C storage. This stabilization had a temporal component however, in that all of the activities analyzed began to decline with increasing storage time at 4°C. These results indicate that although the stabilization of platelets through the manipulation of second messenger systems was an effective method to control storage lesions which occur at 4°C, additional parameters must be controlled to fully exploit this stabilization system.

Platelets stored at 22°C via conventional methods display storage lesions in part due to the metabolic depletion of the energy sources in the plasma which eventually leads to a drop in the pH of the stored platelet suspension. This loss of an energy source can be somewhat overcome in that system by the inclusion of metabolite precursor in the storage buffer. In order to examine if the loss of platelet activity over time which we have observed with the extended storage at 4°C was due to a metabolic depletion event, the stabilization storage solution was evaluated with the addition of metabolic precursors. The TC mixture and the FC mixture, concentrations as described above (Table 8), were evaluated with the addition of acetate (120mM) or the addition of glutamine (2mM), pyruvate (2mM), and glucose (20mM) for the ability to extend the storage time at 4°C. Figure 2 displays the activity profile of platelets stored at 4°C under these conditionswith the above concentration of effectors..

Figure 2. Effects of the Addition of Heparin or Metabolic Additives to the TC and FC Mixtures.







The results of these experiments are as follows:

1) The addition of acetate to either the TC mixture or the FC mixture had no effect on the activity profile.

2) The addition of heparin to the FC mixture enhanced the agonist-induced aggregation but not the HSR at day 7.

3) The inclusion of metabolic precursors to the FC mixture increased all of the platelet activity parameters.

4) The collagen induced aggregation remained at 70% of that of fresh platelets at day 7 for the FC mixture with metabolic additives.

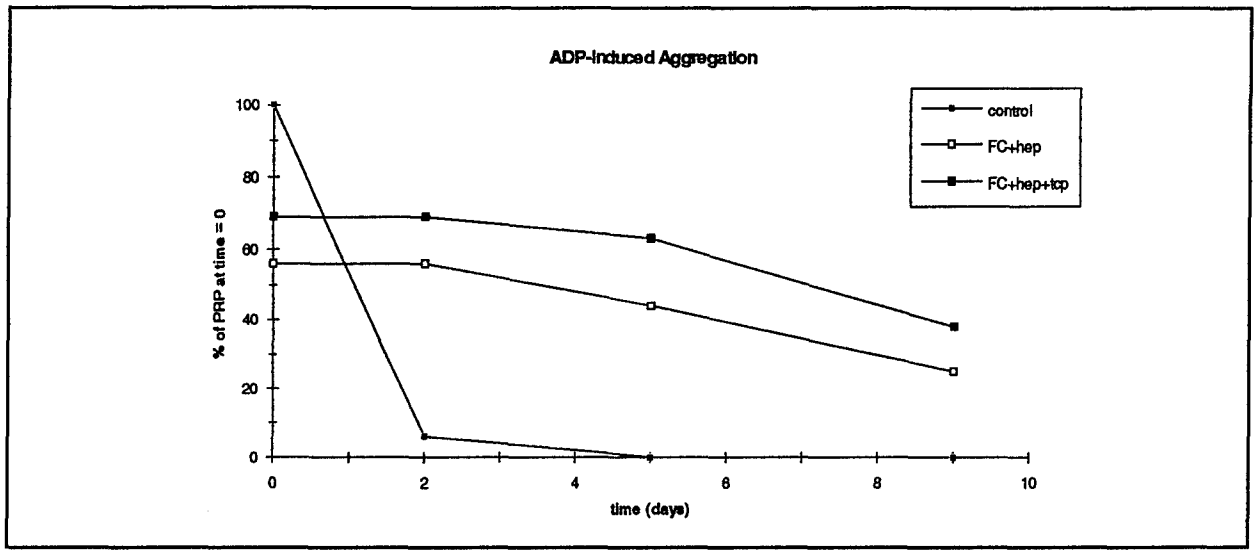
5) The HSR was two-fold higher at day 7 for the FC solution with metabolic additives as compared to the FC mixture alone.

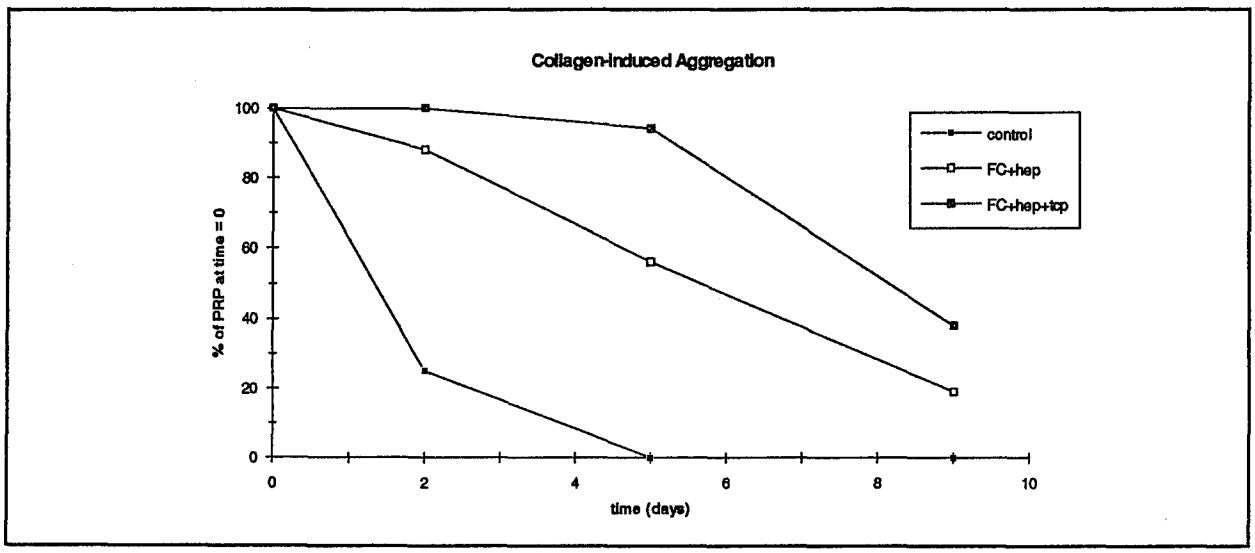
6) A small amount of spontaneous activation was observed in the platelets stored in the FC mixture with metabolic additives (data not shown).

These experiments demonstrated that in addition to the importance of stabilizing the platelets against second messenger activation, it was also necessary to supply critical biochemical metabolites in order to sustain extended storage at 4°C.

Extended 4°C storage periods for platelets were also examined using the second messenger storage system which yielded the best results previously during the shorter exposures. The FC mixture with the addition of heparin and ticlopidine was analyzed for the ability to stabilize platelets for extended storage at 4°C and the results are displayed in Figures 3 and 4. The concentration of the effectors are as described in Table 8.

Figure 3. Effects of the FC Mixture with Heparin and Ticlopidine on Extended Storage of Platelets





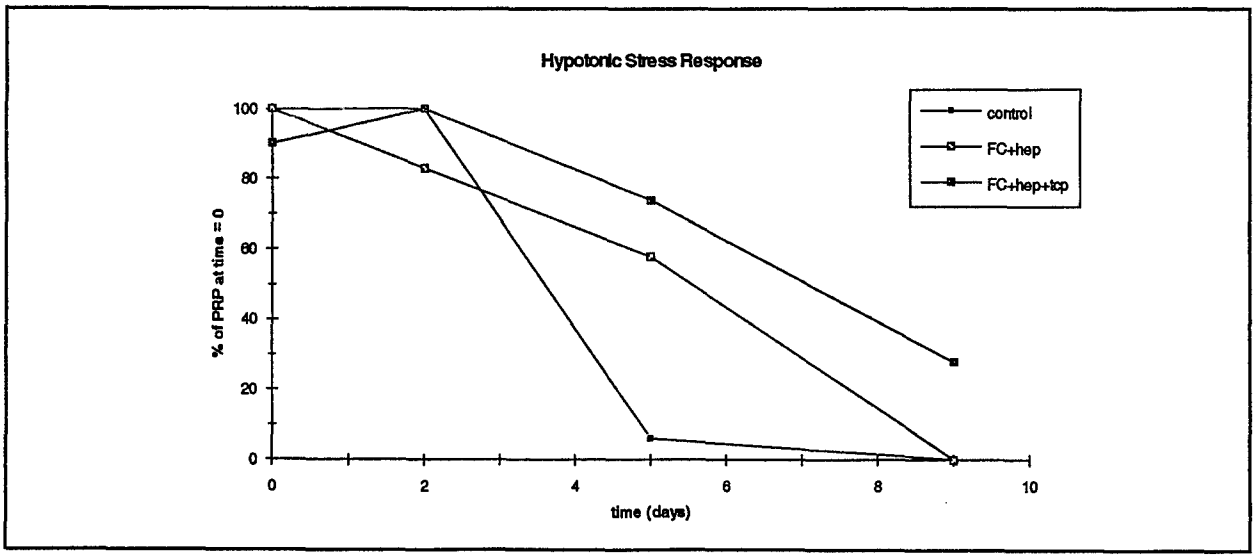
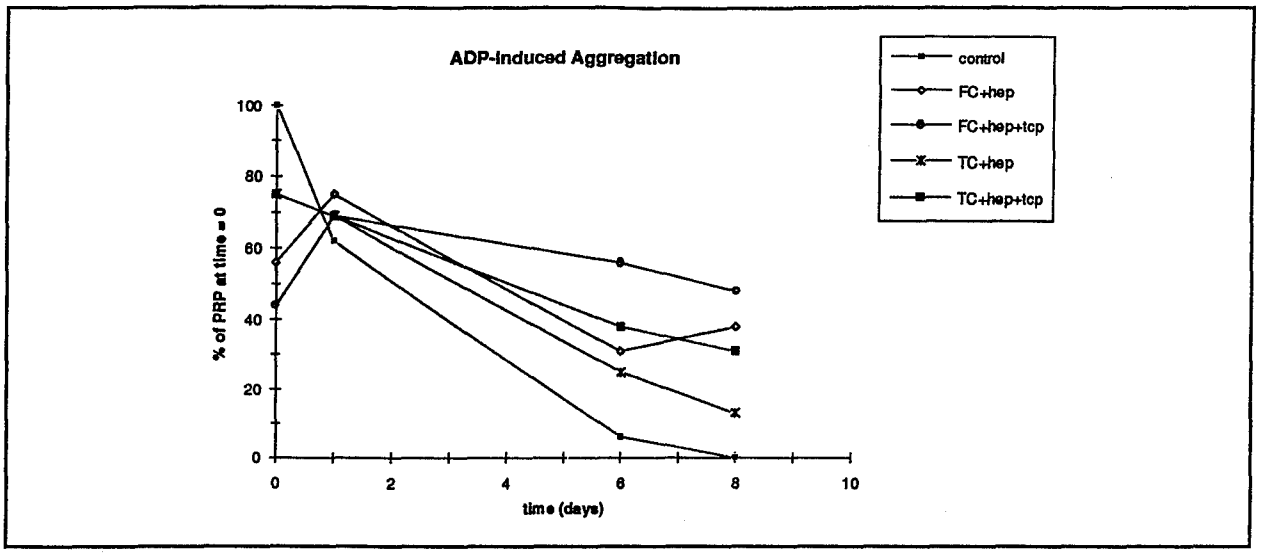
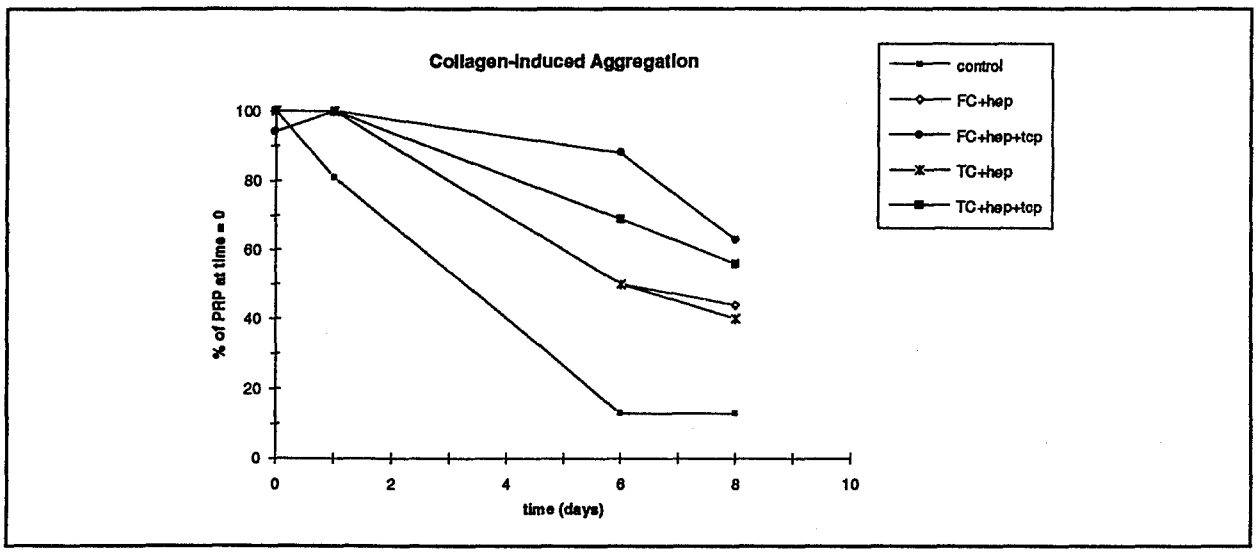
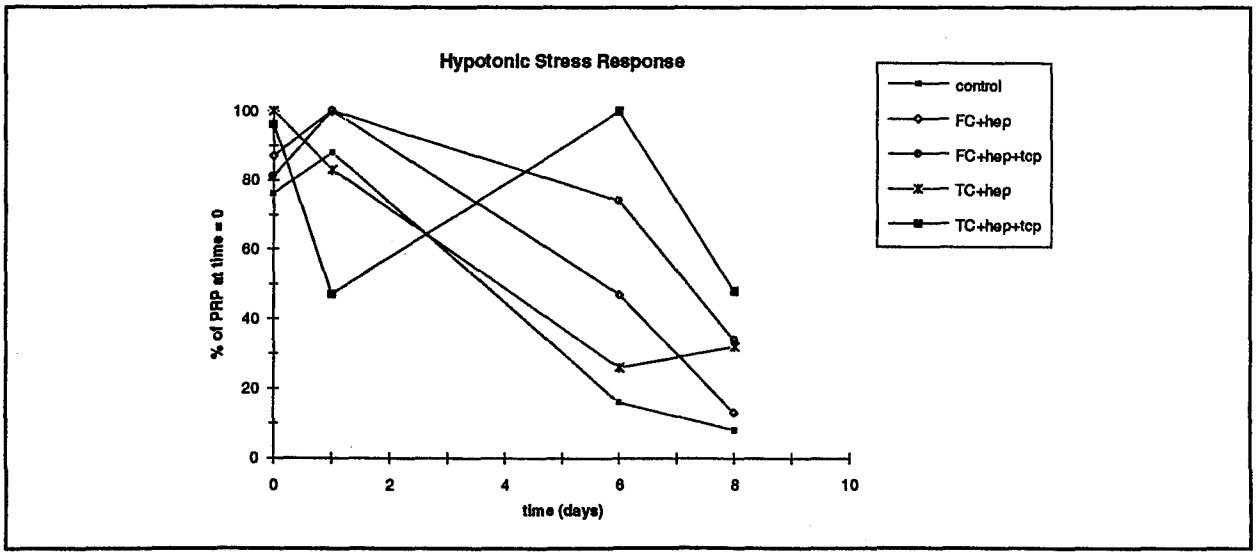


Figure 4. Effects of the Addition of Heparin and Ticlopidine to the TC and FC Mixtures.







The results of these experiments are as follows:

1) The addition of heparin and ticlopidine, both individually and together were beneficial when added to the FC mixture.

2) The second messenger stabilization mixture composed of FC with heparin and ticlopidine yielded platelets at day 6 which displayed activity profiles equivalent to fresh platelets.

3) The platelets stored at 4°C in the 5C mixture with heparin and ticlopidine began to show some loss of activity at day 8 and day 9. These platelets still showed some activity with ~50% ADP and ~60% collagen-induced aggregation and ~30% HSR, as compared to fresh platelets. It should be noted that these results are comparable/superior to those seen with conventional storage at 22°C for 3-5 days.

4) The platelets at day 9 displayed no spontaneous activation and the cell number was 100% of the PRP at the time of acquisition (data not shown).

The conclusion of the experimental series employing second messenger effectors to stabilize platelets for storage at 4°C is that by controlling the biochemical mechanism responsible for storage lesions, platelets can be successfully stored at 4°C. The combination of amiloride, adenosine, sodium nitroprusside, dipyridamole, quinacrine, heparin and ticlopidine added to a platelet concentrate has been demonstrated to stabilize the platelet biochemistry such that the cells can withstand storage at 4°C. Platelets stored under these conditions display activity profiles similar to fresh platelets for as long as six days and still retain as much as 50% activity of fresh platelets by day nine. In addition, there is no loss of cell number during the entire storage period. Moreover, all of these effectors are currently in use for in vivo medicinal applications, at considerably higher concentrations than are being used in this application, indicating that there is a strong possibility that the storage solution presents no hinderance to direct transfusion.

Cryopreservation

Platelet Processing

Previous research, which examined the potential to cryopreserve platelets in the presence of second messenger modifiers, demonstrated that stabilization of the platelet biochemistry yielded freeze-thawed platelets which displayed higher levels of activity then untreated platelets. These experiments employed the TC mixture, controlled rate cooling and storage at -80°C. Following thaw, the cryopreserved platelets yielded greater than 90% recovery of cell number and agonist induced aggregation of 30% and 55% for ADP and collagen, respectively. In contrast the control platelets yielded between 60-70% recovery of cell number and only 10% and 30% aggregation in response to ADP and collagen, respectively. Clearly, as seen with the 4°C storage of platelets, the stabilization of the platelets via treatment with second messenger effectors enhanced the ability of the platelets to resist storage lesions. Based on this data and the information generated in the experimental series analyzing 4°C storage of platelets, the effects of additional second messenger agents and the effects of improved processing methods were examined with respect to the maintenance of the activity profile of cryopreserved platelets.

The first experimental series examining cryopreservation of platelets was to define the physical processing of the platelets during the cryopreservation protocol. In these experiments, platelets were acquired from PRP with and without pretreatment with the second messenger agents. The platelets were then pelleted and enough plasma was removed to yield a 1/10 volume. The platelets were resuspended, a two fold solution of 9% DMSO in plasma was added yielding a final DMSO concentration of 6%, and the final platelet suspension was gently mixed. The platelets were then

slow rate cooled (1°C/min) to -80°C and stored for the indicated time period. The effect of cooling rates on platelet recovery was examined by comparing this slow rate cooling to rapid cooling via a direct liquid nitrogen plunge. Table 9 compares these two cooling rates.

Table 9. Effects of Freezing Rates.

	% of PRP at t=0	
Conditions	ADP Aggregation time (days)	Collagen Aggregation time (days)
Control Rate Freeze Control	12.5	50
TC[1,2]	25	88
TC[3]	50	75
LN_2 Plunge Control	0	0
TC[1,2]	0	0
TC[3]	0	0

[1]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[2]Amiloride added to the whole blood; adenosine and NP added to the PRP. Samples stored in wash buffer
[3]Amiloride added to the whole blood; adenosine and NP added to the PRP. Samples stored in PPP.

An additional comparison analyzed in this experiment is the use of the wash buffer versus the use of plasma. As discussed previously in the 4°C storage section, initial experiments examining platelet storage were performed using a storage buffer as opposed to the autologous plasma. The conclusion from that discussion was that the most efficient and practical methods, for extending platelet storage, should employ plasma as the storage medium. Therefore, both wash buffer and plasma were compared to insure that no deleterious effect would result from this change in procedure. The data in Table 9 shows that rapid cooling via direct liquid nitrogen plunging was detrimental to the platelets'

recovery. In contrast, the conventional slow rate cooling yielded viable platelets, following the freeze-thaw cycle. In addition, the platelets cryopreserved in plasma displayed similar activity profiles to those stored in wash buffer.

The second processing mode analyzed was the method for slow rate cooling. During these experiments a controlled, slow-rate cooling at 1°C/min, using a cell freezer, was compared to the direct insertion of the platelet preparation into a -80°C freezer, which is estimated to cool the sample at 1-2°C/min. Table 10 demonstrates that both methods yielded similar activity parameters for the platelets following cryopreservation. This is important since the ability to directly freeze platelet preparation by insertion into the -80°C freezer is the most efficient and economical method for the application of cryopreservation to unit size platelet preparations.

Table 10. Comparison of Methods for Slow Rate Freezing of Platelets.

	% of PRP at t=0							
Condition	ADP Aggregation time (days)				Collagen Aggregation time (days)			
	1		6		1		6	
	Control Rate	Direct	Control Rate	Direct	Control Rate	. Direct	Control Rate	Direct
Control	29	100	41	71	50	75	56	38
TC[1,2]	100	100	23	33	75	88	75	81
TC[3]	100	88	24	29	75	100	69	88
TC[4]	94	76	29	26	100	100	75	65

Condition	% of PRP at t=0			
	HSR time (days)			
	1		6	
	Control Rate	Direct	Control Rate	Direct
Control	0	5	26	29
TC[1,2]	57	50	35	65
TC[3]	29	18	58	42
TC[4]	32	8	39	56

[1]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[2]Amiloride added to the whole blood; adenosine and NP added to the PRP.
[3]Amiloride, adenosine, and NP added to the PRP.
[4]Amiloride (0.33mM) added to the whole blood; adenosine and NP (concentration as described above added to the PRP.

Table 11. Effects of Storage Temperatures.

Conditions	% of PRP at t=0							
	ADP Aggregation time (days)				Collagen Aggregation time (days)			
	7		13		7		13	
	-80C	-135C	-80C	-135C	-80C	-135C	-80C	-135C
Control	47	47	45	41	59	65	59	65
TC[1,2]	53	56	42	47	71	61	65	59
TC[3]	53	59	45	47	59	67	59	71

[1]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[2]Amiloride added to the whole blood; adenosine and NP added to the PRP.
[3]Amiloride, adenosine, and NP added to the PRP.

The third parameter of platelet processing which was examined was the storage temperature. In these

experiments platelets which had been treated with the second messenger effectors were slow rate cooled in a -80°C freezer, followed by storage at either at -80°C in a conventional low temperature freezer or at -135°C in a liquid nitrogen freezer. The results shown in Table 11 indicate that the platelets stored at both temperatures over a period of 13 days displayed similar recovery of activity following cryopreservation. These results are important since -80°C freezers are more readily available and more economical since they are currently in use at most Blood Centers for the cryopreservation of erythrocytes.

Effects of Second Messenger Reagents

The next area of study involving the cryopreservation of platelets was that of formulating of the optimal second messenger effector stabilization solution. Based on the data developed during the study of 4°C storage, the first question addressed, to assess the optimization of the method of cryopreservation, was to define the approach for the addition of the second messenger agents. In these experiments, the TC mixture was analyzed for the ability to stabilize the platelets during cryopreservation.

Table 12. Effects of the Point of Addition of the Second Messenger Agents.

	% of PRP at t=0		
Conditions	ADP Aggregation	Collagen Aggregation	HSR
Control	66	81	23
TC[1,2]	66	81	35
TC[3]	58	88	69
TC[4]	83	88	36
TC[5]	83	85	55

[1]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[2]Amiloride added to the whole blood; adenosine and NP adde to the PRP.

[3]Amiloride, adenosine, and NP added to the PRP.
[4]Amiloride (0.33mM) added to the whole blood; adenosine and NP added to the PRP.
[5]Amiloride (0.33mM), adenosine, and NP added to the PRP.

The addition of amiloride to the whole blood followed by the other modifiers to the PRP was compared to the addition of all the agents directly to the PRP. In addition, the effects of using a lower concentration of amiloride (0.33 mM) was also analyzed and the results are shown in Table 12.

The results of these experiments demonstrate the following:

1) The addition of the second messenger effectors to the PRP was as effective as adding the amiloride component of the TC mixture to the whole blood.

2) The original concentration of amiloride was slightly better than the lower concentration especially in terms of HSR activity. This is true regardless of the point of addition.

3) The TC mixture of second messenger modifiers protected against loss of activity during the freeze-thaw process, yielding platelets which displayed high levels of activity as compared to fresh platelets.

4) Cryopreserved platelets treated with second messenger effectors gave a recovery of cell number of >95% while the control platelets gave only 60% recovery of cell number (data not shown).

An expansion of the stabilization buffer from the TC mixture to the additional formulation of the FC mixture, containing quinacrine and dipyridamole, improved the activity profile of platelets stored at 4°C. This formulation was examined for the ability to increase the post-thaw activity parameters for cryopreserved platelets. In addition, the effects of heparin on platelet stabilization was also analyzed. Table 13 shows the effects of the TC and the FC mixture with and without heparin on the ability of platelets to be stored via cryopreservation. These results showed that the addition of heparin had no effect, either positive or negative, on the post storage platelet activity. Similar to the 4°C storage results, the inclusion of additional modifiers of the platelet second messenger systems increased the ability of the platelets to withstand storage lesions during storage and thus, displayed

higher levels of activity following the freeze-thaw process.

Table 13. Effects of the Addition of Heparin to the TC and the FC Mixtures.

	% of PRP at t=0				
Conditions[1]	ADP Aggregation time (days)		Collagen Aggregation time (days)		Cell Number
	1	8	1	8	
Control	25	31	87	75	59
TC[2]	69	56	94	69	100
TCw/Hep[3]	63	38	88	57	95
FC[4]	50	88	63	71	100
FC w/Hep[5]	56	38	75	44	88

[1]All of the second messenger effectors were added to the PRP.
[2]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[3]Triple Complex (concentrations as described above) with heparin [0.13mg/ml (20U/ml)].
[4]Five complex consist of TC (concentrations as described above) with dpm (20uM) and quin (0.1uM).
[5]Five Agent Complex (concentrations as described above) with heparin [0.13mg/ml (20U/ml)].

The increases in recovery of platelet activity seen with the FC mixture as compared to the TC mixture were not as large as those seen during 4°C storage. This is probably due to the continued (albeit slow) biochemical process still occurring at 4°C, which eventually overcomes the stabilization effects. These biochemical events are completely arrested at -80°C.

An important issue addressed during the development of the experimental series examining the 4°C storage was the step in the protocol for processing platelets at which the addition of the stabilization solution occurred. In order to best accommodate the current Blood Banking practices, it was concluded that the most advantageous point for the addition of second messenger agents would be to the platelet pellet. Table 14 shows the results of the experiments to examine whether the point of addition of the stabilization solution had any effect on the ability of platelets to recover activity

following cryopreservation. The TC mixture was added either to the PRP or the final platelet pellet and the platelets were then cryopreserved using 6% DMSO as described previously.

Table 14. Effects of the Point of Addition of the Second Messenger Agents.

	% of PRP at t=0							
Conditions	ADP Aggregation time (days)				Collagen Aggregation time (days)			
	0	2	8	15	0	2	8	15
Control	94	35	9	12	94	53	47	41
TC[1,2]	82	76	65	65	100	82	76	71
TC[3]	80	71	61	67	96	82	71	71

Conditions	HSR time (days)				Cell Number
	0	2	8	15	
Control	100	23	5	0	46
TC[1,2]	88	62	30	22	87
TC[3]	76	81	29	26	98

[1]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[2]The TC mixture added to the PRP.
[3]The TC mixture added to the platelet pellet.

The results of Table 14 demonstrate that similar to the 4°C storage of platelets, addition of the second messenger effectors to the final platelet pellet was as effective in preventing storage lesions during cryopreservation as the addition to the PRP. This allows for the integration of this unique cryopreservation method, which employs the stabilization solution, to the current procedures used in conventional blood banking.

To further develop this system of the addition of second messenger reagents directly to the platelet pellet, the effect of the FC mixture was analyzed following this protocol. Table 15 displays the results of this experiment in which the TC mixture was compared to the FC mixture.

Table 15. Effects of the Arachidonic Metabolic Effector with the TC Mixture.

	% of PRP at t=0							
Conditions	ADP Aggregation time (days)				Collagen Aggregation time (days)			
	0	5	8	56	1	5	8	56
Control	83	58	33	25	88	75	50	44
TC[1]	87	67	50	37	94	100	63	48
TC w/Quin[2]	83	75	50	33	88	100	63	50
TC w/Dyp[3]	83	67	52	33	81	100	65	40
FC[4]	50	50	47	33	56	75	44	50

	% of PRP at t=0				
Conditions	HSR time (days)				Cell Number
	0	5	8	56	
Control	100	38	36	37	59
TC[1]	93	48	54	44	84
TC w/Quin[2]	75	68	40	67	87
TC w/Dyp[3]	70	75	71	52	91
FC[4]	85	46	53	74	84

[1]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[2]Triple Complex (concentrations as described above) with quin (0.1uM).
[3]Triple Complex (concentrations as described above) with dpm (20uM).
[4]Five complex consist of TC (concentrations as described above) with dpm (20uM) and quin (0.1uM).

The results of these experiments are as follows:

1) The addition of the FC mixture yielded slightly higher platelet activity than the TC mixture following cryopreservation.

2) Platelets cryopreserved with the FC mixture gave 75% HSR activity as compared to fresh platelets.

3) Long term storage of platelets at -80°C (>50 days) showed no detrimental changes in the platelet activity profile as compared to shorter storage times.

4) Platelets treated with the second messenger agents yielded >85% recovery of cell number, while control platelets gave 60% recovery of cell number.

The second messenger reagent ketorolac, in conjunction with the FC mixture, was also analyzed for the ability to improve the recovery of platelet activity following cryopreservation and the results of this analysis was shown in Table 16.

Table 16. Effects of the Addition of Ketorolac to the FC mixture.

	% of PRP at t=0								
Conditions	ADP Aggregation time (days)			Collagen Aggregation time (days)			HSR time (days)		
	1	2	7	1	2	7	1	2	7
Control	100	33	58	100	59	76	100	10	10
TC[1]	71	65	71	100	82	85	92	19	12
TC w/Quin[2]	83	65	76	100	82	88	82	47	14
TC w/Dyp[3]	73	68	71	100	88	92	85	26	10
FC[4]	82	59	76	100	82	76	92	24	11
FC w/Ktc[5]	76	38	35	82	47	49	80	24	6

[1]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).
[2]Triple Complex (concentrations as described above) with quin (0.1uM).
[3]Triple Complex (concentrations as described above) with dpm (20uM).
[4]Five Agent Complex consist of TC (concentrations as described above) with dpm (20uM) and quin (0.1uM).
[5]Five Complex (concentrations as described above) with ktc (10mM).

Similar to the effects in the 4°C storage of platelets, ketorolac, in conjunction with the FC mixture, was detrimental to the recovery of platelet activity following cryopreservation.

All of the above described cryopreservation experiments followed the only clinically approved method for the use of DMSO in the cryopreservation of platelets. These procedures employ 6% DMSO in plasma, which is added as a two fold volume addition from a 9% DMSO stock. Since the Blood Bank platelet pellet is resuspended to a 1/5 volume (platelet concentrate), the final platelet preparation volume following the addition of the DMSO is 1/1.6 of the original PRP volume. This represents an amount of DMSO which precludes the option of directly transfusing the cryopreserved platelet unit following the thaw process. Platelet preparations cryopreserved by this method must first be washed in order to remove the majority of the DMSO. This wash step is detrimental to the platelets and results in the loss of both platelet cell number and platelet activity.

To overcome the prohibitive effects of the inclusion of large volumes of DMSO, an experiment was designed to analyze the ability to store platelets, via cryopreservation, using 6% DMSO at a final platelet concentration of 1/10 of the original PRP volume. This yields a population of platelets which contain significantly less absolute amounts of DMSO following the thaw procedure. Platelets cryopreserved using this method can be diluted with fresh plasma from the concentrated 1/10 volume to the volume of the conventional method (1/1.6). This process would reduce the DMSO concentration six fold to a final DMSO concentration of 1%. This concentration of DMSO can be safely transfused and thus, the damaging post thaw wash step can be eliminated. To test this protocol, platelets were processed according to this new method. Following the generation of the platelet pellet, all of the plasma was removed. To this pellet 1/10 volume of plasma containing

6%DMSO, with and without the TC mixture, was added and the platelets were resuspended. The platelets were then cryopreserved by the methods used previously. The results of these experiments are shown in Table 17.

Table 17. Effects of Cryopreservation of Platelets at 1/10 Volume in 6% DMSO.

	% of PRP at t=0														
Conditions	ADP Aggregation time (days)					Collagen Aggregation time (days)					HSR time (days)				
	0	2	6	8	27	0	2	6	8	27	0	2	6	8	27
Control	100	62	43	62	50	84	63	74	63	63	21	41	11	51	78
TC[1]	100	56	28	56	35	95	68	68	68	74	20	83	31	70	76

[1]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).

The conclusion of these experiments are as follows:

1) Cryopreservation of platelets at 1/10 volume with DMSO yielded platelets with higher activity following 27 days storage than platelets stored via the conventional cryopreservation method.

2) This cryopreservation method gave >95% recovery of cell number and >75% HSR as compared to fresh platelets.

3) Platelets cryopreserved at 1/10 volume displayed good platelet activity profiles even in the absence of stabilization with the TC mixture.

These results indicate that by using this modification of the current DMSO cryopreservation methods, platelets can be stored at -80°C with high recoverability of platelet activity and cell number. In addition, the low absolute amounts of DMSO makes direct transfusion of this platelet preparation a possibility.

Animal Model

Development of an animal model to examine the in vivo characteristics of platelet preparations subjected to long-term storage is an important component of the platelet preservation study. An animal model will allow for the examination of circulatory half-life and 24 hour survivability of platelets following the storage protocol.

Preliminary studies to find a mammalian animal platelet population that displayed similar characteristics to the human platelets demonstrated that rats were inappropriate as a model animal, while rabbits appeared to represent a viable animal for a model system. Initial experiments showed that the rabbit platelets could be inhibited from aggregating in response to agonist stimulation in a reversible manner, similar to human platelets.

However, experiments conducted during this phase of the platelet preservation study have revealed that rabbits do not represent a viable animal for study due to differences in storage profiles as compared to human platelets. Table 18 shows the activity profile of different animal platelets examined for the ability to mimic human platelets in terms of storage characteristics.

While the rabbit platelets were shown previously to be reversibly inhibited when incubated with the second messenger effectors, these experiments revealed that the control rabbit platelets were unaffected by 4°C storage. In contrast to human platelets which showed storage lesions at 4°C in the absence of second messenger modifiers, the rabbit platelets stored under identical conditions displayed normal activity profiles, following 4°C storage. This inconsistency eliminates rabbits

as a viable animal model since the effects of the second messenger agents can not be analyzed under these conditions.

Table 18. Analysis of Animal Platelets.

Condition	% of PRP at t=0							
	ADP Aggregation time (days)				Collagen Aggregation time (days)			
	0	1	6	10	0	1	6	10
Rabbit[1] Control	100	91	54	36	100	100	77	77
TC[2]	36	18	18	0	62	48	46	46
Goat Control	100				100			
TC[2]	100				86			
Dog[3] Control		38				61		
TC[2]		13				17		

[1]Rabbit platelets were analyzed following storage at 4°C.
[2]Triple Complex consists of amiloride (1mM), adenosine (0.1mM) and NP (25uM)>
[3]Dog platelets were analyzed following cryopresrvation.

Additional animals were then examined for the ability to mimic the in vitro human platelet system under experimentation in this study. Goat platelets were tested and were shown to be totally unreactive to the second messenger effectors (Table 18). The addition of the TC mixture to the goat PRP was unable to inhibit the agonist induced aggregation and thus goat platelets do not qualify as an appropriate model to study the in vivo effects of stored platelets. Canine platelets were examined for compatibility to the human platelet system using the cryopreservation storage system. The results of these experiments (Table 18) showed that the canine platelets do not mimic

human platelets, in that the canine platelets treated with the second messenger effectors displayed significant storage lesions following cryopreservation.

The final animal examined for use as an animal model for in vivo platelet analysis following storage was the baboon. Baboon blood obtained from The Southwest Research Institute (San Antonio, TX) was processed as previously described for human blood. Table 19 shows the activity profiles of these platelets stored at 4°C.

Table 19. Analysis of Baboon Platelets.

	% of PRP at t=0						
Conditions	ADP Aggregation time (days)			Collagen Aggregation time (days)			HSR time (days)
	0	1	5	0	1	5	1
Baboon[1] Control	100	50	71	100	71	100	44
TC[2]	100	43	71	100	65	100	95

[1]Baboon platelets were analyzed following storage at 4°C.
[2]Triple Complex consist of amiloride (1mM), adenosine (0.1mM) and NP (25uM).

The results demonstrated that the baboon platelets treated with second messenger effectors resisted storage lesions during the 4°C storage. This is especially evident in the two fold higher HSR of the TC mixture treated platelets as compared to the control platelets. In addition the second messenger agent treatment yielded 100% recovery of cell number, which is similar to the recovery seen with human platelets. The conclusion from these experiments is that the baboon represents the best possibility for an animal model which mimics the human platelets in term of storage characteristics.

It should be noted at this point that preliminary discussions with the Red Cross have indicated that it may not be necessary to employ an animal model to examine the in vivo profile of stored platelets. An alternative scenario was presented in which a toxicity study of the final second messenger agent mixture would be performed to demonstrate that the mixture is non-toxic following transfusion. Once non-toxicity is established, a pilot clinical study of the stored platelets would be performed with human subjects. The possibility of using this approach to evaluate the in vivo characteristics of the platelets is currently under review.

Summary

4°C Storage of Platelets

-Addition of second messenger agents effectively stabilized platelets against storage lesions when added to the platelet pellet (platelet concentrate).

-Platelets incubated with the TC mixture yielded 100% recovery of cell number.

-Platelets stored at 4°C with the TC mixture displayed signs of storage lesions by day 5.

-The arachidonic acid cascade inhibitors, quinacrine and dipyridamole, both effectively and reversibly inhibited platelet activation.

-The addition of quinacrine and dipyridamole to the TC mixture yields a five component (FC) mixture which enhanced the stabilization of platelets during 4°C storage as compared to the TC solution alone.

-The addition of the anti-thrombotic agent ketorolac to the FC mixture was ineffective in increasing the platelets' resistance to storage lesions.

-The addition of calcium channel blockers was ineffective in inhibiting agonist-induced aggregation.

-The addition of heparin to both the TC and the FC mixture increased the post storage activity profiles of platelets.

-The addition of the platelet aggregation inhibitor ticlopidine to the second messenger effector solution enhanced the stabilization of platelets during 4°C storage.

-The addition of the metabolic precursor acetate to the second messenger modifier mixtures was ineffective in increasing the platelet activity parameters following storage at 4°C.

-The addition of the metabolic precursors pyruvate, glutamine and glucose, extended the 4°C storage time at which platelets could recover full activity.

-The FC mixture with the addition of heparin and ticlopidine represented the optimal stabilization buffer examined to date to allow extended storage of platelets at 4°C.

-Platelets, treated with the FC mixture with heparin and ticlopidine, displayed activity profiles, at day 6, equivalent to fresh platelets.

-Platelets incubated for 9 days at 4°C with the FC solution with heparin and ticlopidine displayed ~50% ADP- and ~60% collagen-stimulated aggregation and ~30% hypotonic stress response as compared to fresh platelets. These results compare favorably to conventional 22°C storage at 5 days which gave ~30% ADP and ~80% collagen activation response and ~60% hypotonic stress response.

Cryopreservation of Platelets

-Controlled rate cooling of platelets (1°C/min) was the optimal method for freezing platelets for storage. In contrast, rapid freezing (liquid nitrogen plunge) caused 100% loss of platelet activity.

-Slow rate cooling by direct insertion of the platelet preparation into a -80°C freezer (~1-2°C/min) was as effective for cryopreservation as control rate freezing.

-Storage of platelets, treated with the second messenger effectors and cryopreserved with 6% DMSO was as effective at -80°C, in a standard low temperature freezer, as at -135°C in a liquid nitrogen freezer.

-The addition of the second messenger agents to the platelet pellet (platelet concentrate) effectively stabilized platelets against storage lesions following cryopreservation.

-The addition of the TC stabilization mixture to cryopreserved platelets yielded >95% recovery of cell number.

-The use of the FC stabilization mixture enhanced the platelet activity profiles following the thaw process as compared to the TC mixture.

-Platelets cryopreserved with the FC mixture gave >90% recovery of cell number, ~35% ADP and ~50% collagen stimulation, and ~75% HSR activity as compared to fresh platelets.

-Long term storage at -80°C of second messenger treated platelets (>50 days) showed no increase in storage lesion over time.

-The use of a cryopreservation method which froze the platelet preparation at 1/10 volume decreased the absolute amount of DMSO in the thawed platelet solution which eliminated the need for a post thaw wash step.

-This new cryopreservation method yielded >95% recovery of cell number and >75% HSR as compared to fresh platelets.